UNCLASSIFIED

AD NUMBER

AD422369

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Administrative/Operational Use; Sep 1963. Other requests shall be referred to U.S. Army Biological Labs., Frederick, MD.

AUTHORITY

BORL D/A ltr, 27 Sep 1971

THIS PAGE IS UNCLASSIFIED

UNCLASSIFIED

AD 422369

DEFENSE DOCUMENTATION CENTER

FOR

SCIENTIFIC AND TECHNICAL INFORMATION

CAMERON STATION, ALEXANDRIA, VIRGINIA

UNCLASSIFIED

NOTICE: When government or other drawings, specifications or other data are used for any purpose other than in connection with a definitely related government procurement operation, the U. S. Government thereby incurs no responsibility, nor any obligation whatsoever; and the fact that the Government may have formulated, furnished, or in any way supplied the said drawings, specifications, or other data is not to be regarded by implication or otherwise as in any manner licensing the holder or any other person or corporation, or conveying any rights or permission to manufacture, use or sell any patented invention that may in any way be related thereto.

422369

TECHNICAL MANUSCRIPT 58

OBSTACLES TO TRANSFORMATION PRESENTED BY BACTERIAL ANATOMY

SEPTEMBER 1963

TISIA B

UNITED STATES ARMY
BIOLOGICAL LABORATORIES
FORT DETRICK

U.S. ARMY BIOLOGICAL LABORATORIES
Fort Detrick, Frederick, Maryland

TECHNICAL MANUSCRIPT 58

OBSTACLES TO TRANSFORMATION PRESENTED BY BACTERIAL ANATOMY

Irving L. Miller

Otto E. Landman

Medical Bacteriology Division
DIRECTOR OF BIOLOGICAL RESEARCH

Project 1C022301A067

September 1963

A portion of the work reported here was performed under Project 4B11-02-065, "Viral and Rickettsial Agent Research," Task -02, "Viral and Rickettsial Agent Laboratory Research." The expenditure order was 2200605. This material was originally submitted as manuscript 5106.

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this document from DDC.

Foreign announcement and dissemination of this document by DDC is limited.

The information in this document has not been cleared for release to the public.

ABSTRACT

Transformation is generally thought to be facilitated by removal or weakening of the cell wall. Newly developed techniques for rescuing and growing (presumptive) protoplasts were used to test this assumption with the Bacillus subtilis transforming system.

DNA from an indole$^+$ strain was mixed with competent cells of B. subtilis strain 168 (indole$^-$) in transforming medium containing 0.5 M sucrose and 10^{-4} M EDTA. Lysozyme was added to samples from this transforming cell suspension at successive time intervals. Twenty to 40 minutes after lysozyme addition, when protoplasting in the samples was complete, the protoplasts and bacillary control suspensions were plated. In samples protoplasted soon after DNA addition, a sharp loss of transformants occurred (10- to 20-fold loss) in the course of protoplast formation. Protoplasts formed prior to DNA addition were completely refractory to transformation. By contrast, when lysozyme was added 120 minutes after the DNA, protoplasting no longer caused a marked loss of transformants. Throughout these experiments, survival of protoplasts (which emerge as L-colonies) ranged from 60 to 100 per cent.

The results are consistent with the hypothesis that, in an early stage of transformation, the DNA resides outside the cell membrane. Removal of the cell wall appears to make subsequent mechanisms for transport of the DNA into the cell inoperative.

OBSTACLES TO TRANSFORMATION PRESENTED BY BACTERIAL ANATOMY

Ever since the discovery that bacteria produce extracellular proteins, the question has been posed as to how macromolecules pass the bacterial membrane. As long as only the passage of macromolecules from the inside of the cell to the outside was being considered, it was usually possible to attribute release of macromolecules to lysis. Since it has been realized, however, that transformation represents the inward passage of large macromolecules into cells that remain viable, the idea that the destruction of the membrane accompanies macromolecular traffic across the cell membrane has had to be discarded. Unfortunately, no very plausible substitute mechanism has been proposed, to our knowledge, except for pinocytosis — but there is no evidence for the existence of pinocytosis in bacteria.

Despite the unsatisfactory theoretical state of this problem, many investigators have proceeded with experiments that are based on the idea that removal of the cell wall would facilitate entry of DNA into cells. Such experiments have repeatedly met with success. Fraser, Mahler, and collaborators[1] have shown that urea-degraded phage particles, incapable of injecting their DNA into intact cells, can infect penicillin spheroplasts of Escherichia coli. Analogous observations were made by Spizizen and collaborators.[2] Cohen and colleagues[3] have demonstrated in marker rescue experiments that bacteriophage T4 DNA can gain entry into E. coli spheroplasts under conditions where intact cell controls do not exhibit any uptake. Evans, Mackal, and Coleman[4] have shown that both λ DNA and T1 DNA can transform E. coli spheroplasts. Finally, American,[5] French,[6] German,[7] and Japanese[8] groups have all shown that spheroplasts permit the entry of phage ϕx 174 DNA while intact E. coli cells either do permit any entry or are less efficient as hosts.

When, during the past year, quantitative techniques, became available in our laboratory[9] for growing protoplasts of Spizizen's transformable indole-deficient strain of Bacillus subtilis,[10] we decided to follow a similar experimental approach, and to demonstrate transformation in these wall-less bodies. In these experiments, our procedure is essentially that used by Young and Spizizen[11] to render cells competent. The cells are grown on a rich agar medium overnight, then transferred to minimal medium with ample trytophan and casein hydrolyzate supplements and allowed to grow for four hours. At this point, again following Young and Spizizen, the cells are diluted 1:10 into minimal medium supplemented with marginal amounts of trytophan and casein hydrolyzate. However, we add 0.5 M sucrose, 0.01 M magnesium sulfate, and 10^{-4} M EDTA to this latter medium to serve as osmotic and chemical stabilizers for protoplasts. After 90 minutes of incubation in this sucrose-stabilized medium, the cells are competent for transformation — we normally obtain about 0.2 per cent transformants. If, instead of adding DNA, we add lysozyme, the cells are converted to protoplasts.

In 20 minutes, more than 95 per cent of the cells can no longer give rise to bacillary colonies. These protoplasts are not dead, however. If they are diluted through stabilizing fluid and plated on special media containing 0.5 M sodium succinate, essentially all of them survive to form L-colonies. Table I shows a medium suitable for the recovery and growth of B. subtilis L-forms. Trytophan must be added for the growth of strain 168. If it is omitted, colonies of tryptophan$^+$ transformants can be scored on this medium, be they L-colonies or bacillary colonies. Each of the L-colonies can be kept in the L-state or can be reverted to the bacillary state by the use of appropriate media.

TABLE I. MEDIUM FOR GROWTH OF PROTOPLAST AND TRANSFORMANTS

Sodium Succinate, pH 7.0	0.5 M
$MgCl_2$	0.005 M
K_2HPO_4	3.5 grams/liter
KH_2PO_4	1.5 grams/liter
Glucose	2.0 grams/liter
Acid-Hydrolized Casein*	1.0 gram/liter
Agar	9.0 grams/liter
Defibrinated Horse Serum	2.5 milliliters/liter
* Can be replaced by NH_4NO_3	1 gram/liter

The results of our first attempts to transform protoplasts of B. subtilis are shown in Table II. Column A shows transformation in sucrose-EDTA-stabilized Spizizen transforming medium in the absence of lysozyme. In the experiment of Column B, DNA was added first, followed 20 minutes later by lysozyme. In the experiment shown in Column C, lysozyme was added 20 minutes prior to the DNA; in Column D both reagents were added simultaneously. Control platings of the protoplast suspensions on trytophan-supplemented media of the Column B, C, and D experiments gave recoveries ranging from 72 to 100 per cent of the original bacillary count. As can be seen from Table II, the preformed protoplasts did not transform at all under the conditions of this experiment and there was very little transformation when DNA and lysozyme were added simultaneously (Column D).

It is the experiment in which lysozyne was added 20 minutes after the DNA that appears to provide a clue as to what may be happening.

It appears that the number of transformants, which is normal at the time of lysozyme addition, actually drops by as much as a factor of 10 as lysozyme incubation is continued. Transformants that seemed to have been established at 20 minutes are actually lost later as a result of lysozyme attack.

Several subsequent experiments have confirmed this original finding. One of these is shown in Table III. If lysozyme is added very shortly after the DNA, 90 to 95 per cent of the transformants are lost; if lysozyme addition is delayed, the loss of transformants is gradually reduced until, 105 minutes after DNA addition, the drop in transformants caused by protoplasting is no longer marked. At this point, the transformations are fixed. Our interpretation of these results is that during the course of its entry into the cell, the transforming DNA temporarily occupies a site on the outside of the cell membrane or in the wall. Then, when the cell wall is removed, this DNA is prevented from completing the entry process. At present, tracer experiments are in progress that are designed to show whether the transforming DNA is released into the supernatant by lysozyme action or whether it remains bound to the protoplasts.

We have performed control experiments to eliminate trivial alternative explanations of our results. For instance, we have shown that loss of transformants probably cannot be attributed to inhibition of transformation by lysozyme, since very large populations of viable protoplasts that have been washed to remove lysozyme also fail to transform. Furthermore, abundant transformation is observed on our L-form medium when a bacillary inoculum is plated with transforming DNA, but few or no transformants are obtained with an L-inoculum under the same conditions.

So far, our results have provided only negative evidence concerning the mode of entry of DNA macromolecules into the cell. These negative results, and considerations based on other phases of our research, have led us to a speculative model about how the DNA might normally cross the membrane barrier. This model is shown in Figure 1.

In the intact cell, the DNA lodges at the membrane near an infolding of the membrane such as a mesosome. As the membrane grows, the DNA is swallowed up by the cell interior. Electron microscope studies suggest* that in protoplast formation the mesosomes and membrane infoldings are lost, much as a fold in an inner tube disappears when the tire is removed. Hence, the transport mechanism for DNA fails in protoplasts.

In Gram-negative spheroplasts, the presence of other wall layers presumably prevents loss of membrane infoldings,[12] leaving the DNA transport mechanism intact.

---

* Ryter, A., and Landman, O.E. Unpublished data.

TABLE II. PER CENT TRANSFORMANTS IN LYSOZYME-TREATED AND CONTROL POPULATIONS OF B. SUBTILIS

First Reagent -	A DNA	B DNA	C Lysozyme	D DNA + Lysozyme
Second Reagent Added at 20 min -		Lysozyme	DNA	
Plating Time Minutes				
0	0.015	0.014	0.0000	0.0064
20	0.19	0.22	0.0000	0.0001
60	0.38	0.042	0.0000	0.0001
120	0.89	0.027	0.0000	0.0000

TABLE III. FIXATION OF TRANSFORMATION TOWARD LYSOZYME REVERSAL

Minutes After DNA Addition	Number of Transformants, 10^{-4} A Plated for Transformants at Time Shown	B Lysozyme Added At Time Shown and Plated for Transformants 40 min Later	$\frac{A}{B}$	Per Cent Survival As L-Colonies
5	0.22	0.018	12.2	80
35	1.7	0.38	4.5	72
70	6.8	0.87	7.8	100
105	10.0	5.0	2.0	56

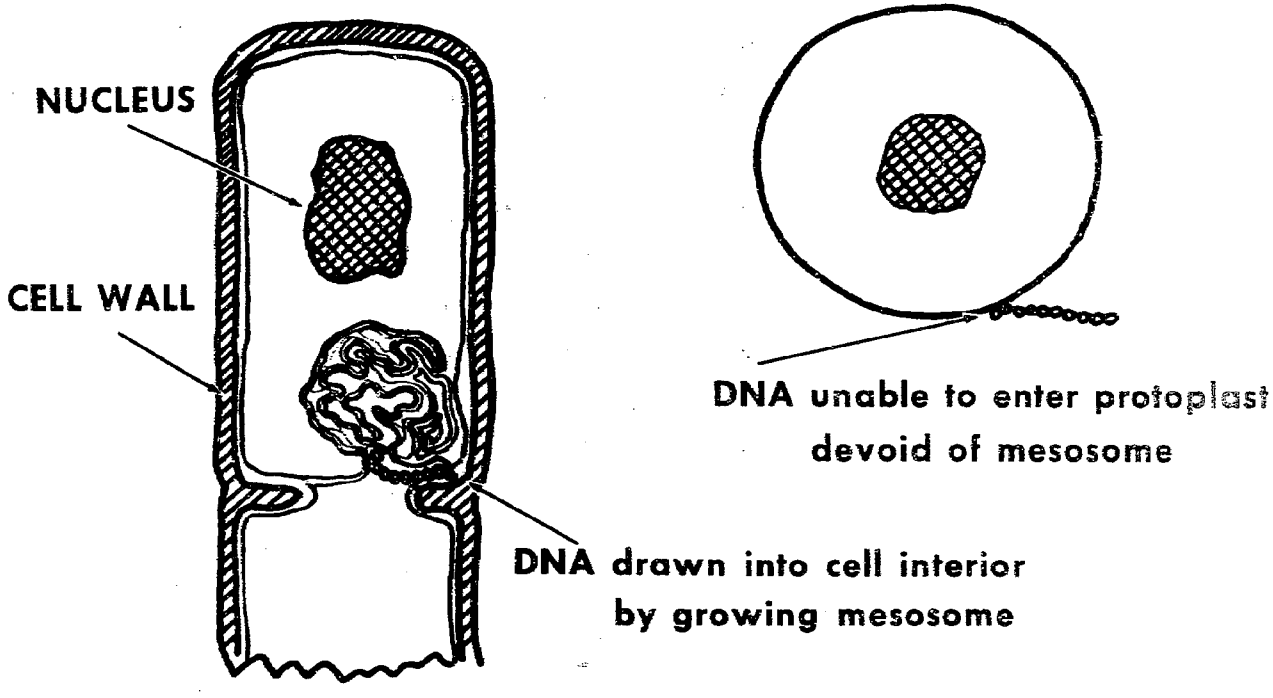

Figure 1. Speculative Models of DNA Entry into Intact Cell, and Failure of DNA to Enter a Protoplast.

LITERATURE CITED

1. Fraser, D.; Mahler, H.R.; Shug, A.L.; and Thomas, C.A., Jr. "The infection of subcellular E. coli strain B with a DNA preparation from T2 bacteriophage," Proc. Natl. Acad. Sci. 43:930-947, 1957.

2. Spizizen, J. "Infection of protoplasts by disrupted T2 virus," Proc. Natl. Acad. Sci. 43:694-701, 1957.

3. Van De Pol, J.H.; Veldhuisen, G.; and Cohen, J.A. "Phage transformation: A new criterion for the biological activity of bacteriophage DNA," Biochim. Biophys. Acta 48:417-418.

4. Evans, E.A.; Mackal, R.P.; and Coleman, L. "Infection of protoplasts with DNA from the virulent bacteriophage T_1," Fed. Proc. 21:456, 1962.

5. Guthrie, G.D., and Sinsheimer, R.L. "Infection of protoplasts of E. coli by subviral particles of bacteriophage ϕx 174," J. Mol. Biol. 2:297-305, 1960.

6. Wahl, R.; Huppert, J.; and Emerique-Blum, L. "Production de phages pardes protoplastes bacterieus infectes par des preparations d' acide desoxyribonucleique," Compt. Rend. Acad. Sci., Paris 250:4227, 1960.

7. Hofschneider, P.H. "Ueber ein infektioeses desoxyribonucleinsaeure-agens aus dem Phagen ϕx 174," Z. Naturforsch. 15b:441, 1960.

8. Sekiguchi, M.; Taketo, A.; and Takagi, Y. "An infective DNA from bacteriophage ϕx 174," Biochim. Biophys. Acta 45:199, 1960.

9. Landman, O.E., and Halle, S. "Induction of inherited changes in bacteria by enzyme treatment," Proc. VIII Internatl. Cong. Microbiol., 1962. p. 28.

10. Spizizen, J. "Transformation of biochemically deficient strains of Bacillus subtilis by deoxyribonucleate," Proc. Natl. Acad. Sci. 44:1072, 1958.

11. Young, F.E., and Spizizen, J. "Physiological and genetic factors affecting transformation of Bacillus subtilis," J. Bacteriol. 81:823, 1961.

12. Landman, O.E.; Burchard, W.K.; and Angelety, L. "Lysogeny and bacteriophage adsorption in stable and reverting L-forms of Salmonella paratyphi B and E. coli," Bacteriol. Proc., 1962. p. 58.